Nonstable staphylococcus aureus small-colony variants are induced by low pH and sensitized to antimicrobial therapy by phagolysosomal alkalinization

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Abstract: BACKGROUND: Staphylococcus aureus-infected patients treated with antibiotics that are effective in vitro often experience relapse of infection because the bacteria hide in privileged locations. These locations include abscesses and host cells, which contain low-pH compartments and are sites from which nonstable S. aureus small-colony variants (SCVs) are frequently recovered. METHODS: We assessed the effect of low pH on S. aureus colony phenotype and bacterial growth, using in vitro and in vivo models of long-term infection. RESULTS: We showed that low pH induced nonstable SCVs and nonreplicating persisters that are capable of regrowth. Within host cells, S. aureus was located in phagolysosomes, a low-pH compartment. Therapeutic neutralization of phagolysosomal pH with ammonium chloride, bafilomycin A1, or the antimalaria drug chloroquine reduced SCVs in infected host cells. In a systemic mouse infection model, treatment with chloroquine also reduced SCVs. CONCLUSIONS: Our results show that the acidic environment favors formation of nonstable SCVs, which reflect the SCVs found in clinics. They also provide evidence that treatment with alkalinizing agents, together with antibiotics, may provide a novel translational strategy for eradicating persisting intracellular reservoirs of staphylococci. This approach may also be extended to other intracellular bacteria.

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Non-stable *Staphylococcus aureus* small colony variants are induced by low pH and sensitized to antimicrobial therapy by phagolysosomal alkalinization

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Abstract

Background: Despite treating patients with in vitro effective antibiotics, *Staphylococcus aureus* infections often relapse because they hide in privileged locations such as abscesses or host cells. Both, abscesses and host cells, feature compartments characterized by low pH and from both non-stable *S. aureus* small colony variants (SCVs) are frequently recovered.

Methods: We assessed the effect of low pH on *S. aureus* colony phenotype and bacterial growth, using in vitro and in vivo long-term infection models.

Results: We showed that low pH induced non-stable SCVs and non-replicating persisters, capable of regrowth. Within host cells, *S. aureus* was located in phagolysosomes, a low pH compartment. Therapeutic neutralization of phagolysosomal pH with ammonium chloride, bafilomycin A1, or the anti-malaria-drug chloroquine reduced SCVs in infected host cells. In a systemic mouse infection model, treatment with chloroquine also reduced SCVs.

Conclusions: Our results show that the acidic environment favors formation of non-stable SCVs, reflecting SCVs found in clinics. They also provide evidence that alkalinizing agents together with antibiotics may provide a novel translational strategy for eradicating intracellular persisting staphylococcal reservoirs. This approach may also be extended to other intracellular bacteria.

Introduction

The human pathogen *Staphylococcus aureus* colonizes approximately a third of all humans and is one of the leading causes of bacteremia and infective endocarditis in the industrialized world [1]. In addition to emerging antibiotic resistance, persisting and recurrent infections substantially add to morbidity and mortality [2, 3]. Recurrence rates, in particular after osteomyelitis or endocarditis, are high and infections may relapse even years after apparent cure [4]. Infection recurrence is associated with non-stable SCVs and/or non-replicating persisters for several reasons [5-8]. Their arrested or slow growth and reduced metabolism renders antibiotics inefficient [9-13]. Moreover, they preferentially hide in privileged locations such as in abscesses and within host cells [14-17]. These privileged locations shield them from the host's innate immune system and from extracellularly active antibiotics. In addition, antibiotics do not penetrate abscesses efficiently and are less active due to the low pH [18-22]. Therefore the 'ubi pus ibi evacua' – the necessity of surgical removal of abscesses formulated in the antiquity still applies nowadays despite highly active antibiotics. In contrast to abscesses, intracellular bacteria cannot be mechanically removed and often resist eradication by currently available antibiotics.

Most SCVs isolated in clinical microbiology laboratories revert to the large colony phenotype upon sub-cultivation. Due to these non-stable properties, stable genetically-modified SCV mutants with defects in the electron transport system have been used to find new strategies to target SCVs and have been found to localize in host cell lysosomes [23]. However, stable SCVs only partially reflect the clinical SCVs. They are less virulent [24] and do not revert.

Due to the non-stable characteristics, clinical SCVs have not been studied so far in detail. It remains unclear how SCVs are induced and maintained in patients. We established conditions mimicking the host environment in which non-stable non-replicating persisters and/or SCVs are found. Low pH induced reversible persisters and/or SCVs. Low pH adapted bacteria persisted within lysosomes by cessation of growth resulting in SCVs on agar plates. In contrast to the

stable genetically modified SCVs used so far, low pH-induced non-stable SCVs reverted to the highly virulent and fast-growing form, reflecting SCVs isolated in clinics which are most likely responsible for causing recurrent infections. Staphylococcal SCVs and/or intracellular persisters were reduced in vivo as well as in vitro by combination therapy consisting of phagolysosome alkalinization plus antibiotics. These findings indicate generally applicable novel therapeutic strategies against intracellularly persisting *S. aureus*.

Materials and Methods

Cell lines, bacterial strains and culture conditions

The human lung epithelial carcinoma cell line A549 [25] was purchased from ATCC (ATCC CCL-185). The cells were maintained in our laboratory and grown in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% FBS (Fetal Bovine Serum, GE Healthcare) at 37 °C in 5 % CO_2.

The *Staphylococcus aureus* strains used in this study are listed in Table 1. *S. aureus* was grown in LB Lennox broth (BD Biosciences) at 37 °C, shaking or on Columbia agar + 5 % sheep blood plates (BioMérieux).

Media with adjusted pH (pH 4.0, 5.5, 6.5 and 7.4) were prepared with DMEM (4.5 g/ml Glucose, without Phenolred, Pyruvat and L-Glutamine, Life Technologies) supplemented with 10 % FBS and 4 mM of L-Glutamine (Life Technologies). The pH 4.0, 5.5 and 6.5 media were prepared with buffers (containing Na_2HPO_4 and citric acid, both from Fluka) of pH 2.6, 4.0 and 5.6, respectively and were mixed at a ratio of 3:7 with DMEM. pH 7.4 was maintained by 50 mM HEPES (Life Technologies).

pH dependent SCVs induction assay

S. aureus colonies were picked and used to inoculate the defined pH media (pH 4.0, 5.5, 6.5 and 7.4) to a starting optical density of 0.2 at 600nm (OD_{600}) and further incubated without shaking at 37 °C in 5 % CO_2 for up to 7 days. The pH and the OD_{600} of cultures were measured daily. To enumerate viable bacteria, culture aliquots were removed, serial-fold diluted and plated on blood agar plates.

Colony forming units (CFUs) were enumerated after overnight incubation at 37 °C. The colony phenotype was determined by morphological analysis after an additional overnight incubation at room temperature. Small colony variants (SCVs) were identified based on their size (one tenth the size of normal colonies), reduced pigmentation and reduced hemolysis.

To detect non-replicating persisters, bacteria were stained with DsRed-tagged *S. aureus* cell wall binding domains (CBDs). CBDs bind to the bacterial cell wall with high affinity and allow the tracking of peptidoglycan synthesis and bacterial replication [26]. In brief, bacteria were mixed with 0.1 mg/ml DsRed-CBDs in PBS (50 mM NaH_2PO_4, 120 mM NaCl, pH 6), washed and added to the defined pH media for further cultivation. Before visualization, cultures were washed and resuspended in PBS pH 8.0.

Intracellular persistence model

A549 cells (1 x 10^6) were seeded into the wells of a 12 well plate in 1 ml medium. Cells were infected as described previously with minor modifications [14]. Briefly, cells were infected with *S. aureus* Cowan at a multiplicity of infection (MOI) of 1 or 100 for intracellular persistence assays and microscopy, respectively. Three hours post-infection, cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS, Life Technologies) and Flucloxacillin (Actavis, 1 mg/ml in DMEM + 10 % FBS) was added in DMEM for 2 hours to kill any extracellular bacteria. Supernatants were monitored for sterility by plating an aliquot on agar plates. Flucloxacillin-containing media were supplemented with lysosomotropic alkalinizing agents (20 µM chloroquine, 1 nM bafilomycin A1, 5 mM ammonium chloride, all from Sigma). Flucloxacillin-

containing media without lysosomotropic alkalinizing agents served as controls. The washing step was repeated and fresh medium was added to the cells daily.

After extensive washing with DPBS, host cells were lysed (0.08 % Triton X-100 from Fluka in DPBS) and serial-fold dilutions of the cell lysates were plated onto blood agar plates to detect intracellular persisting bacteria. CFUs were enumerated and the colony phenotype determined.

To assess cell damage induced by *S. aureus* or by lysosomotropic alkalinizing agents, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed as previously described [27]. Non-infected, non-treated cells served as 100 % cell viability control.

Fluorescence staining and confocal microscopy

Bacteria were labeled with Carboxy-Fluorescein diacetate Succinimidyl Ester (CFSE, Life Technologies) prior to host cell infection according to manufacturer's instructions. Infected cells were fixed with 4 % paraformaldehyde (EMS), permeabilized with 0.2 % Triton X-100 and blocked with 2 % bovine serum albumin (Sigma). Lysosomes were visualized with mouse anti-human LAMP2-CD107b primary antibody (BD Biosciences) and with Alexa Fluor 594-conjugated goat anti-mouse IgG secondary antibody (Life Technologies). Purified Mouse IgG1 κ was used as isotype control (BD Biosciences). DNA was stained with 4',6-Diamidin-2-phenylindol (DAPI, Sigma). Samples were visualized at room temperature by confocal microscopy (CLSM SP5, Leica) and a 63x objective (HCX PL APO lambda blue 63.0x1.40 OIL UV) using Leica LAS AF software. Images were processed using Imaris (version 7.1.1), and Photoshop (Adobe). Deconvolution was done with Huygens software (SVI).

To assess the pH of phagolysosomes, A549 cells treated with flucloxacillin only or supplemented with lysosomotropic alkalinizing agents were stained with 5 µg/ml acridine orange (Sigma) for 15min and analyzed by confocal microscopy [28].

Scanning Electron Microscopy

Bacteria grown on agar plates or in liquid media were fixed with 2.5 % glutaraldehyde in DPBS and harvested onto glass slides using a cytospin (3 min, 1000 rpm). After incubation in 1 % osmium tetroxide, bacteria were dehydrated with an increasing concentration of ethanol (70-100%), critical-point dried (Bal Tec CPD 030, Leica) and coated with 5 nm of platinum in a sputtering device (Bal Tec SCD 500, Leica). The samples were examined with a field emission scanning electron microscope (SUPRA 50 VP, Zeiss) using the in-lens detector.

Transmission Electron Microscopy

Infected A549 cells were fixed with 2.5% glutaraldehyde and 1% formaldehyde in DPBS. After incubation in 1 % osmium tetroxide and staining with 1% uranyl acetate, cells were embedded in resin. Ultrathin sections (70 nm in thickness) were prepared using an ultramicrotome (Ultracut E, Reichert), collected on slot copper grids and stained with Reynolds lead citrate. The sections were examined using a Philips CM100 transmission electron microscope equipped with a side mounted digital camera (4k x 3k, Gatan).

Ethics Statement

Mice were purchased from Janvier, France, and handled in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol 140/2011 was approved by the Institutional Animal Care and Use Committee of the University of Zurich. All efforts were made to minimize suffering of animals employed in this study.

Mouse infection model

C57BL/6 wild-type mice (8-11 weeks old) were infected intraperitoneally (ip) with *S. aureus* Cowan (10^8 CFUs). Eight hours post-infection, mice were treated once with flucloxacillin (1 mg ip) alone or with chloroquine (0.2 mg ip [29]) in addition. Chloroquine (0.2 mg ip) was redosed

after 48 hours. Three days post-infection, mice were euthanized and organs were harvested for bacterial counts.

Results

pH-dependent induction of *S. aureus* Small Colony Variants (SCVs)

The well-defined MSSA strains 6850 and Cowan and MRSA strain JE2 were grown in 4.0, 5.5, 6.5 and 7.4 pH media, mimicking the pH found in physiologic sites such as lysosomes, abscesses and blood. Directly after inoculation *S. aureus* showed a large colony phenotype, independent of the pH. The frequency of SCVs significantly increased over time in pH 4.0 growth medium and reached 39% (JE2 and 6850) and 28% (Cowan) after five days (**Figure 1A**). In contrast, pH 7.4 growth medium sustained SCVs below 2% in all strains tested (**Figure 1A**). An intermediate percentage of SCVs was found at pH 5.5 and 6.5. (Total colony counts and absolute SCV numbers are shown in **Supplementary Figure 1**). Thus, we showed a clear correlation between low pH and non-stable SCV formation. This method permitted easy and controlled formation of non-stable, non-genetically modified SCVs in various *S. aureus* strains.

Induction of non-replicating *S. aureus* by low pH

The pH-dependent growth of *S. aureus* Cowan was followed over time by labeling the bacterial cell wall with fluorescent cell wall binding domains (CBDs) [26, 30]. Immediately after staining, bacterial cell walls were fully labeled (control, **Figure 1B I**). After three days in pH 4.0 growth medium, the majority of bacteria still exhibited fluorescent cell walls, consistent with absent bacterial replication (**Figure 1B II**). In contrast, bacteria grown at pH 7.4 proliferated extensively, demonstrated by highly fragmented and reduced fluorescent cell wall labeling (**Figure 1B III**). Scanning electron microscopy (SEM) of bacteria originating from a small colony (**Figure 1C)**, obtained under low-pH conditions, showed impaired cell division resulting in rod-shaped *S. aureus*, in contrast to large colony bacteria showing normal cell division (**Figure 1C, arrows**).

Growth resumption of low pH-adapted *S. aureus* persisters

Our findings indicated that both, SCVs and non-replicating persisters, are induced by low pH. In clinical practice, the presence of these persisting bacteria correlate with increased recurrence of infection which implies that bacteria revert to a highly virulent and fast-growing form [14]. We therefore tested in vitro whether low pH-induced SCVs and/or non-replicating persisters can restore normal growth in neutral pH. Non-replicating *S. aureus* persisters were induced and kept at pH 4.0 for three days and then transferred to pH 4.0, 5.5, 6.5 or 7.4 growth media. Bacteria in pH 7.4 and 6.5 resumed growth after approximately 12 hours (**Figure 1D**) whereas bacteria kept at low pHs (< 6.5) remained in a non-proliferating state (**Figure 1D**). The bacteria transferred to pH 7.4 growth medium had a large colony phenotype after eleven hours (**Supplementary Figures 2A-C**). These data suggest that both persisting phenotypes were reversible adaptions to low pH as supported by the capability of regrowth and phenotype switch to the fast-growing form upon neutralization of pH.

Intracellular induction of *S. aureus* SCVs

We investigated whether internalized *S. aureus* exhibited a SCV phenotype. MSSA strain Cowan is highly invasive, but not cytotoxic [31] which allowed maintaining this strain intracellularly over several days in the lung epithelial cell line A549 [27]. Extracellular bacteria were killed by adding a high dose of flucloxacillin to the infected host cells. Flucloxacillin is typically used to treat *S. aureus* endocarditis in patients. Absence of extracellular bacteria was confirmed by sterility of culture supernatants. Host cells were lysed to release intracellular bacteria and colony counts, as well as colony phenotypes, were determined at various time points. Five hours after infection, 0.2% of all viable intracellular bacteria had a SCV phenotype (**Figure 2A**). The number of viable intracellular persisting bacteria decreased during the course of infection while the frequency of SCVs increased and reached 5.6% after seven days (for absolute SCV numbers see **Supplementary Figure 3**).

Phagolysosomal localization of persisting *S. aureus*

Our data indicated that acidity favored SCV formation, suggesting that the acidic phagolysosomal milieu may have the same effect. A549 cells were infected with *S. aureus* Cowan. Intracellular bacteria were localized within LAMP-2 antibody positive vesicles (**Figure 2B**), visualized by fluorescence microscopy. LAMP-2 (CD 107b) is highly expressed in phagolysosomes, suggesting that intracellular persisting *S. aureus* predominantly resided within phagolysosomes. Samples stained with IgG1 κ isotype control were negative.

Reduction of *S. aureus* SCVs through phagolysosomal alkalinization

Since low pH induced SCVs and/or non-replicating *S. aureus* and medium pH neutralization resulted in bacterial regrowth, we treated infected host cells with lysosomotropic alkalinizing agents. Chloroquine, bafilomycin A1 or ammonium chloride all neutralized the phagolysosomal pH (see **Supplementary Figures 4A-D**). Host cells treated with flucloxacillin plus lysosomotropic alkalinizing agents exhibited significantly lower percentages of SCVs seven days after infection (**Figure 3A**). No differences in total colony counts between flucloxacillin and flucloxacillin plus alkalinizing agents treated cells were observed (see **Supplementary Figure 5A**). Lysosomotropic alkalinizing agents did not inhibit bacterial growth at the concentrations used (the minimal inhibitory concentrations of chloroquine, bafilomycin A1 and ammonium chloride were above 15.534 mM, 1 µM and 200 mM, respectively, for *S. aureus* Cowan). Absolute SCV numbers are shown in **Supplementary Figure 5B**. No significant differences in host cell viability were observed after treatment with the alkalinizing agents (see **Supplementary Figure 6**).

Growth resumption and reduction of SCV percentages by chloroquine in cells and in mice

Growth resumption of *S. aureus* persisters through treatment of host cells with chloroquine was assessed. Fluorescence microscopy revealed that *S. aureus* localized within phagolysosomes in both, flucloxacillin and flucloxacillin plus chloroquine-treated host cells, three days post-infection

(see **Supplementary Figure 7**). We observed no dividing bacteria in infected host cells without chloroquine treatment. However, chloroquine facilitated bacterial cell division (**Figure 3B**) as assessed by transmission electron microscopy (TEM).

Mice infected with *S. aureus* Cowan were treated with flucloxacillin alone, or with flucloxacillin plus chloroquine (**Figure 4A**). Chloroquine treatment significantly reduced the frequency of SCVs in mice in various organs (**Figure 4B)** and compartments (**Figure 4C**). Absolute bacterial numbers were comparable, independent of chloroquine treatment (see **Supplementary Figure 8A**). For absolute SCV numbers see **Supplementary Figure 8B**.

Discussion

This study showed that low pH, as found in abscesses and within lysosomes, induced the persisting *S. aureus* subpopulations SCVs and non-replicating persisters. Raising pH in the culture medium or within the phagolysosomes using alkalinizing agents reverted *S. aureus* to normal growth.

SCV formation was shown to be triggered by antibiotic pressure [32]. In addition, extreme environmental stresses such as prolonged exposure to low temperature, very acidic or alkaline environments, or osmotic stress may trigger SCV and/or persister formation in *S. aureus* and coagulase-negative staphylococci [33]. These observations, together with our new findings, show how multiple stimuli lead to *S. aureus* persister formation. Localization within the host cell shields *S. aureus* from commonly used antibiotics such as the extracellularly active beta-lactams with poor cell penetration [34]. In addition, the low intraphagolysosomal pH renders antibiotics with intracellular activity such as clindamycin and fluoroquinolones less active [20, 35]. We found that the addition of lysosomotropic alkalinizing agents to the usually prescribed antibiotics such as flucloxacillin reduced the frequency of *S. aureus* SCVs in vitro as well as in vivo. We thus identified a simple strategy to circumvent the host-dependent component of *S. aureus* persister formation. In clinical settings, the presence of SCVs in osteomyelitis and device-related

infections has been associated with increased relapse rates, despite administration of antibiotics [5, 35, 36]. Bacteria adapt to antibiotic stress by SCV and/or persister formation [32]. We now showed that *S. aureus* SCVs and non-replicating persisters retained the ability to revert to a highly virulent and fast-growing form. The capacity to revert to fast growth (phenotype switching) results in relapsing infection. In addition, it renders identification of SCVs difficult. Further aggravating the SCV problem is the underestimation of SCVs in clinical microbiology laboratories, since they form tiny and thus difficult to detect colonies which are easily overgrown by their fast growing counterparts [37].

We postulate that the addition of alkalinizing agents to the usually prescribed antibiotics will reduce the frequency of SCVs and could therefore reduce recurrence rates in the future. Chloroquine should also be considered in combination with vancomycin, a drug that has been shown to have reduced activity against SCVs [38]. This could, at least in part, explain the high relapse rates associated with vancomycin [39]. However, this strategy cannot eradicate extracellularly persisting bacteria as found in abscesses or biofilms as chloroquine acts specifically on lysosomes. Surgery and antibiotics will remain the treatment of choice for these.

Even though we show a clear correlation between low pH and non-stable SCV formation, the underlying mechanism remains to be elucidated. Whether low pH acts as a trigger and induces the formation of new SCVs or whether low pH rather acts as a selective pressure and enriches preexisting SCVs is unclear. Yet, the therapeutic strategy of phagolysosomal alkalinization employed acts on SCVs in general, i.e. on the preexisting SCVs, as well as on newly formed SCVs.

Persisting bacteria are not unique to *S. aureus* but have also been described to occur in various other human pathogens, such as *Salmonella* spp., *Pseudomonas aeruginosa*, *Escherichia coli* and *Mycobacterium tuberculosis* [5, 40-42]. In addition to low pH, bacterial persisters can arise due to mechanisms that include the toxin-antitoxin systems [43, 44]. Accordingly, activation of a SOS response (ppGpp) in response to DNA damage due to oxidative stress results in decreased

ATP levels [45, 46]. This leads to the shutdown of metabolism resulting in reduced growth. Various toxin-antitoxin modules are activated by acidification and/or nutrient starvation in *Salmonella*, causing formation of persisters [47]. In accordance with our findings, *Salmonella* persister formation has been reported in macrophages triggered by the acidic and nutritionally poor environment of the *Salmonella*-containing vacuole that was reversible by addition of bafilomycin A1 [47]. In contrast to bafilomycin A1, chloroquine is routinely used in patients to treat malaria as well as some rheumatic diseases. Phagolysosomal pH neutralization with chloroquine may therefore provide a novel therapeutic eradication strategy against intracellular persisting staphylococcal reservoirs. Already, phagolysosomal alkalinization by chloroquine has proven to be an effective strategy for eradicating the obligate intracellular bacterium *Coxiella burnetii*, the pathogen responsible for Q fever [48]. Phagolysosomal alkalinization restored the activity of intracellular antibiotics resulting in *Coxiella burnetii* clearance. It is tempting to propose that our findings may be applicable to other intracellularly persisting bacterial infections.

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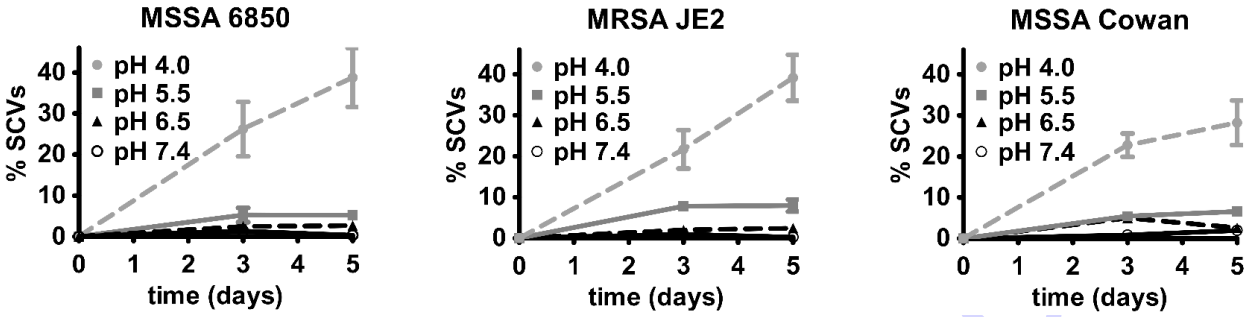
Figure Legends

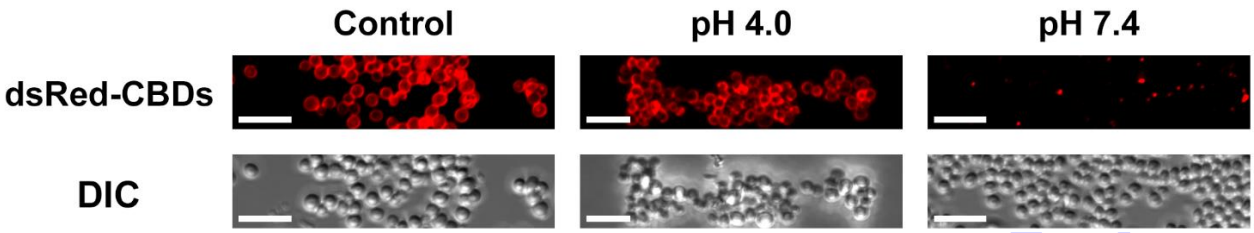
Figure 1. Induction of *S. aureus* SCVs and non-replicating persisters by low pH and bacterial regrowth through pH increase. (A) *S. aureus* strains 6850, JE2 and Cowan were inoculated in media buffered at different pH as indicated. Colony phenotypes of viable bacteria were determined and the percentage of SCVs plotted over time. Three independent experiments done in triplicates are presented as mean ± SEM. **(B)** DsRed-CBDs labeled *S. aureus* Cowan was visualized immediately after staining (Control, I) and three days after inoculation in pH 4.0 (II) and pH 7.4 (III) medium. Respective differential interference contrast (DIC) microscopy images. Scale bars 5 µm. **(C)** Colony phenotype of *S. aureus* Cowan small- (arrow) and large-colony variants. Scale bar 1 mm. SEM analysis of bacteria originating from a small- and large-colony, respectively. Scale bars 1 µm, arrows indicate incomplete bacterial cell division. **(D)** Low pH-induced *S. aureus* persisters were re-inoculated in various buffered pH media as indicated and growth was followed over time. Three independent experiments done in triplicates presented as mean ± SEM.

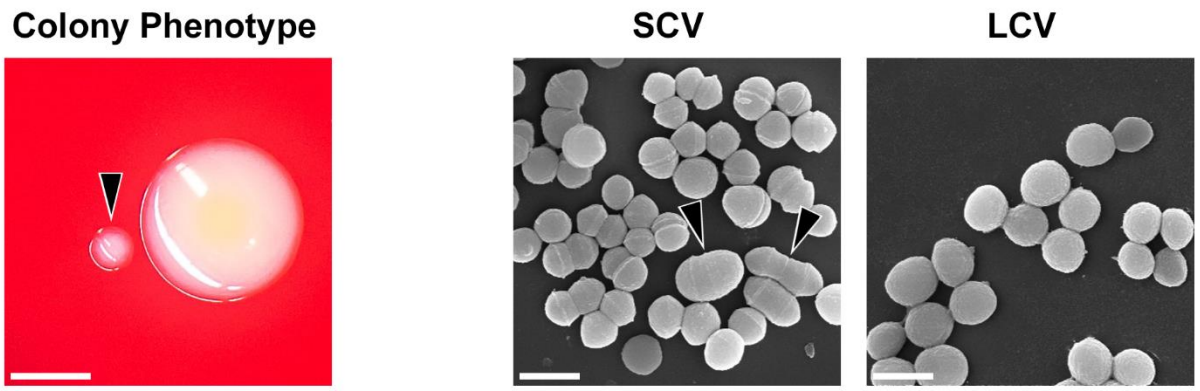
Figure 2. Intracellular persistence of *S. aureus* within phagolysosomes. A549 cells were infected with *S. aureus* Cowan and extracellular bacteria were killed by addition of flucloxacillin [1 mg/ml]. **(A)** The number and phenotype of viable intracellular persisting bacteria were determined at indicated time points. Data are pooled from two experiments performed in triplicates, mean ± SEM. **(B)** The intracellular localization of persisting bacteria (CFSE, green) was analyzed by fluorescence microscopy. Lysosomes were visualized with LAMP-2 antibody (Alexa Fluor 594, red) and nuclei were stained with DAPI (blue). Scale bars 20 µm and 5 µm, respectively.

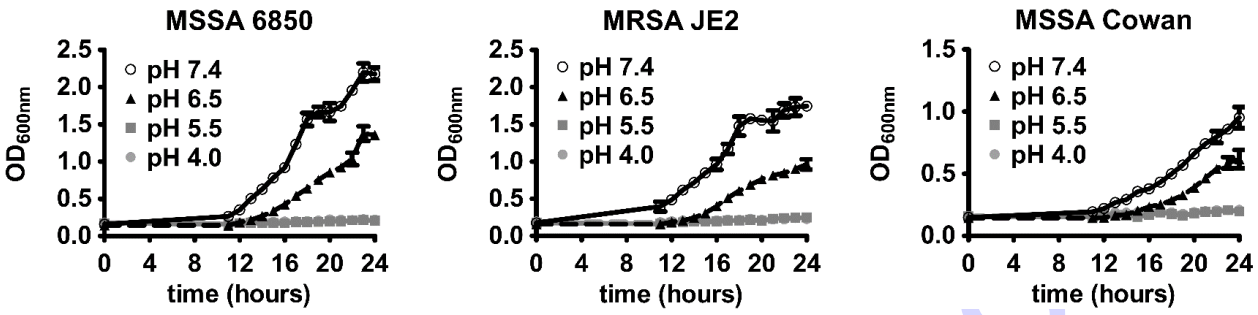
Figure 3. Reduction of *S. aureus* persisters through phagolysosome alkalinization and bacterial cell division after chloroquine treatment. *S. aureus* Cowan-infected A549 cells were treated with flucloxacillin alone (F) or with flucloxacillin plus lysosomotropic alkalinizing agents (CQ, chloroquine; Baf A1, bafilomycin A1; and AC, ammonium chloride). **(A)** Colony phenotypes of viable intracellular persisting bacteria were determined and enumerated at indicated time points. Data were pooled from three independent experiments done in triplicates, mean ± SEM. Two-way ANOVA found the factors time and treatment to be significant (p-value < 0.01). **(B)** Visualization of flucloxacillin (F) and flucloxacillin plus chloroquine (F + CQ) treated cells three days post-infection by TEM. Arrows indicate bacterial cell division. All scale bars 2 µm.

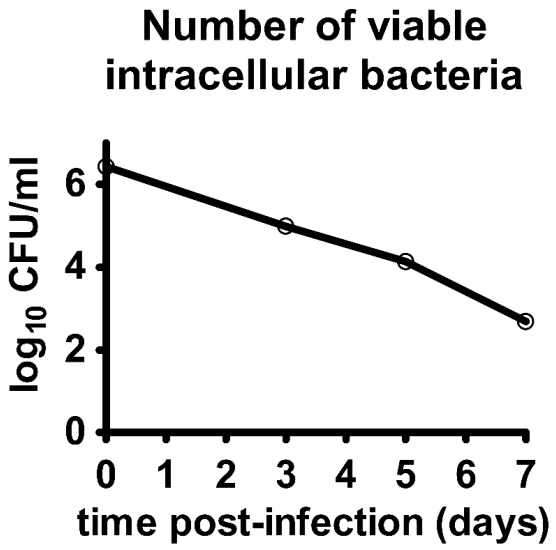
Figure 4. Reduction of *S. aureus* persisters by chloroquine in an in vivo infection model. **(A)** Mice were infected with *S. aureus* Cowan intraperitoneally. Six hours and two days post-infection mice were treated with 1 mg flucloxacillin (F) or with 1 mg flucloxacillin plus 0.2 mg chloroquine (F + CQ). †, sacrifice. Colony phenotypes of bacteria recovered from target tissues **(B)**, peripheral blood and peritoneal lavage **(C)** were determined and enumerated. Each point represents one mouse. Horizontal bars indicate mean ± SEM, $n = 11$ mice per group. PL, peritoneal lavage. Two-way ANOVA found the factor treatment to be significant (p-value < 0.01).

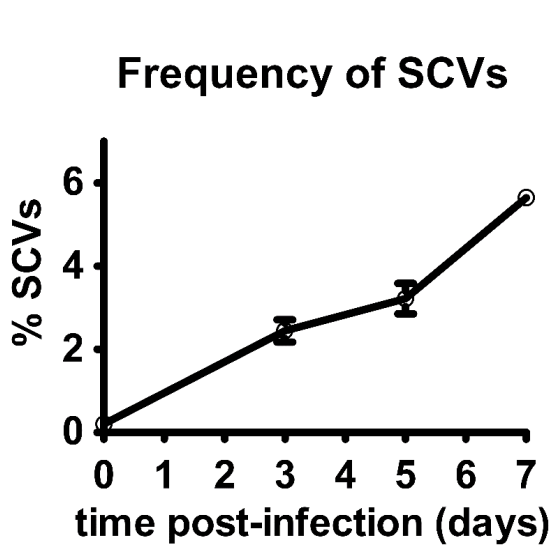


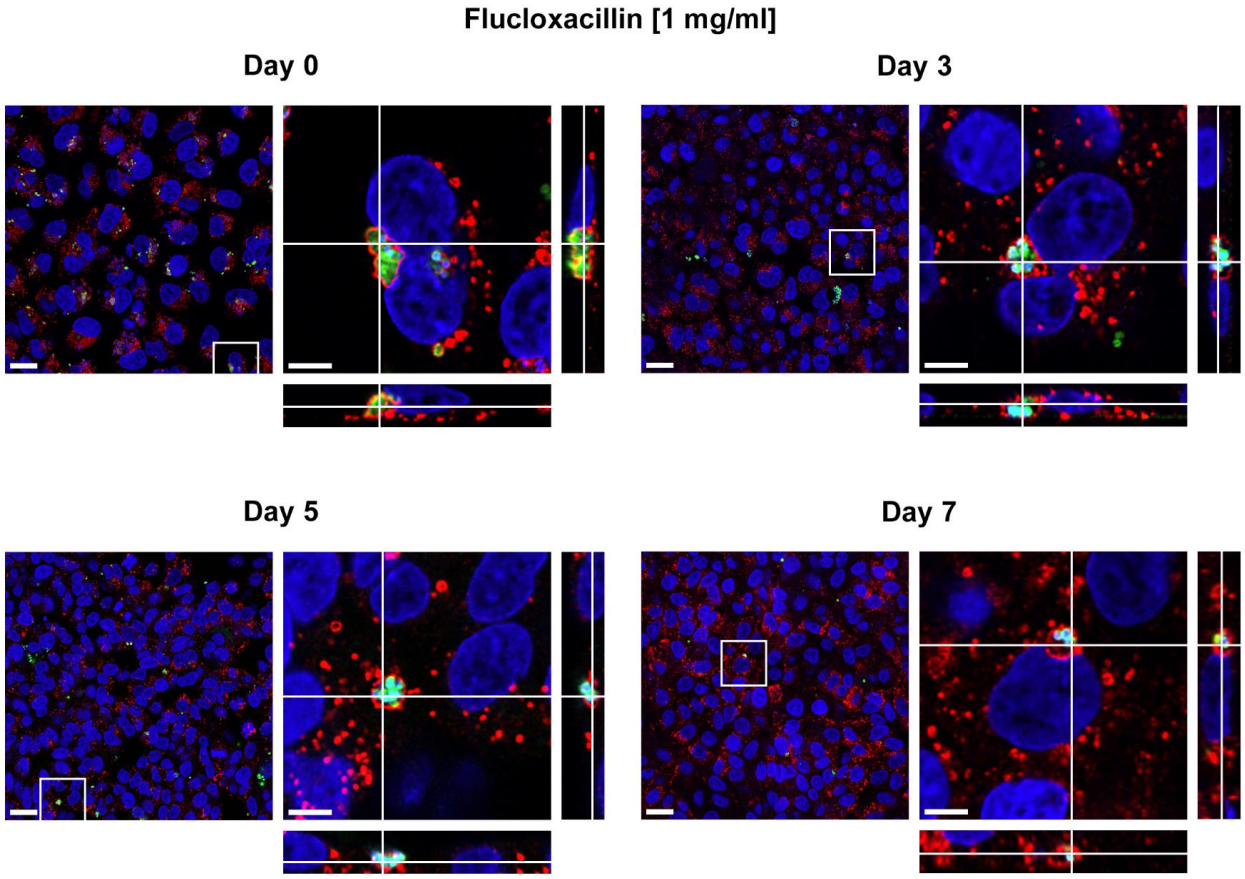


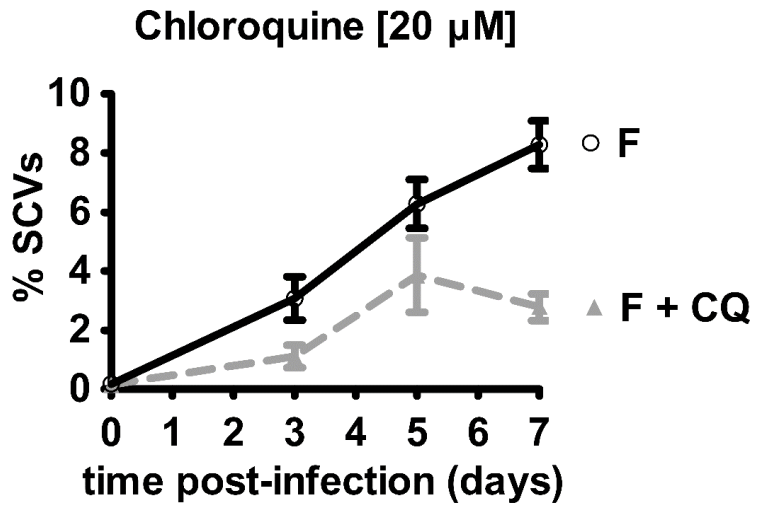


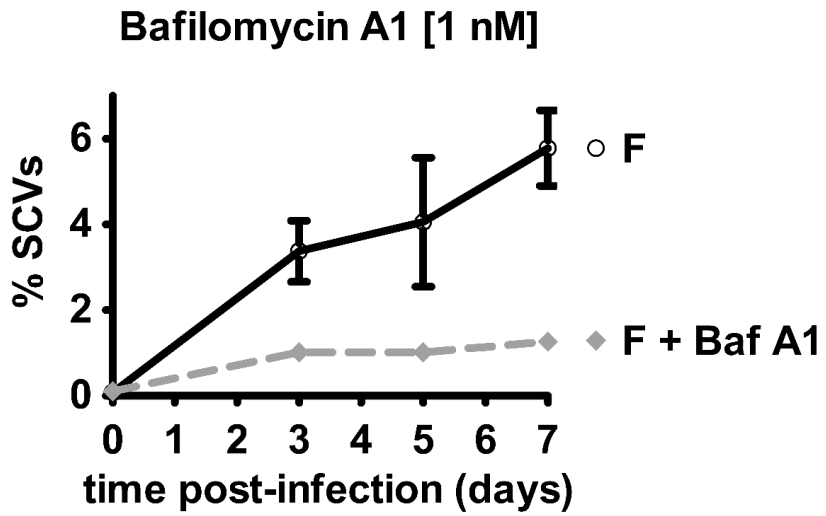


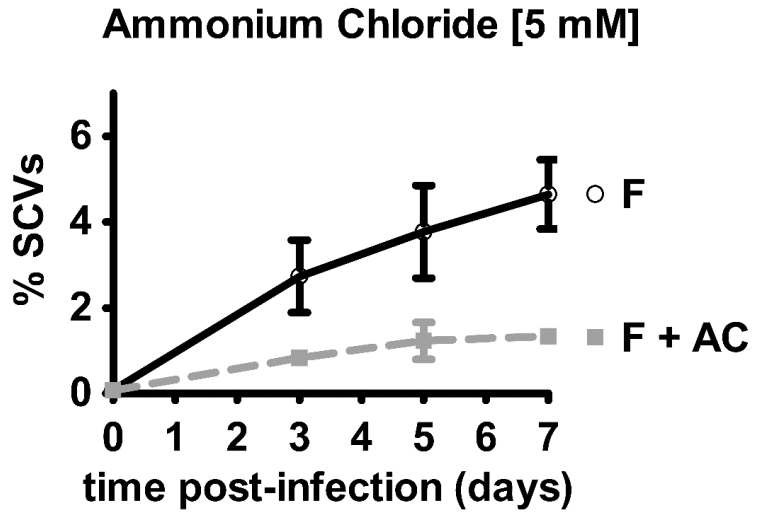


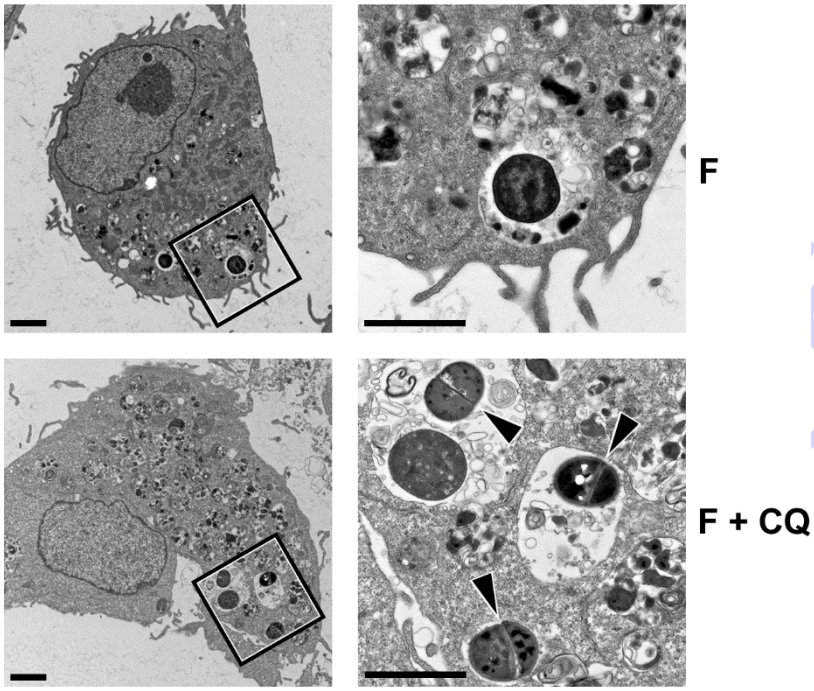


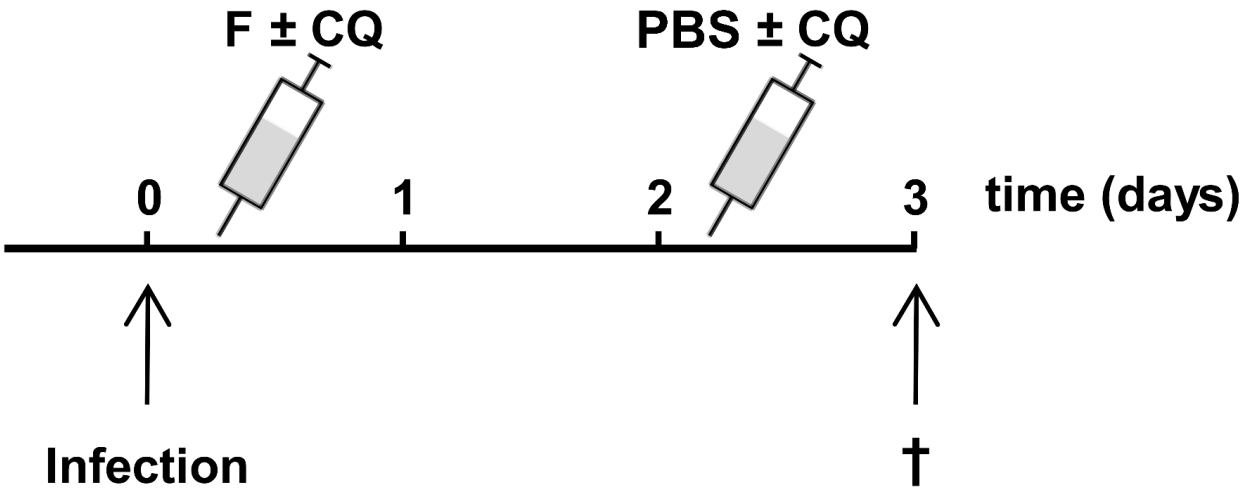


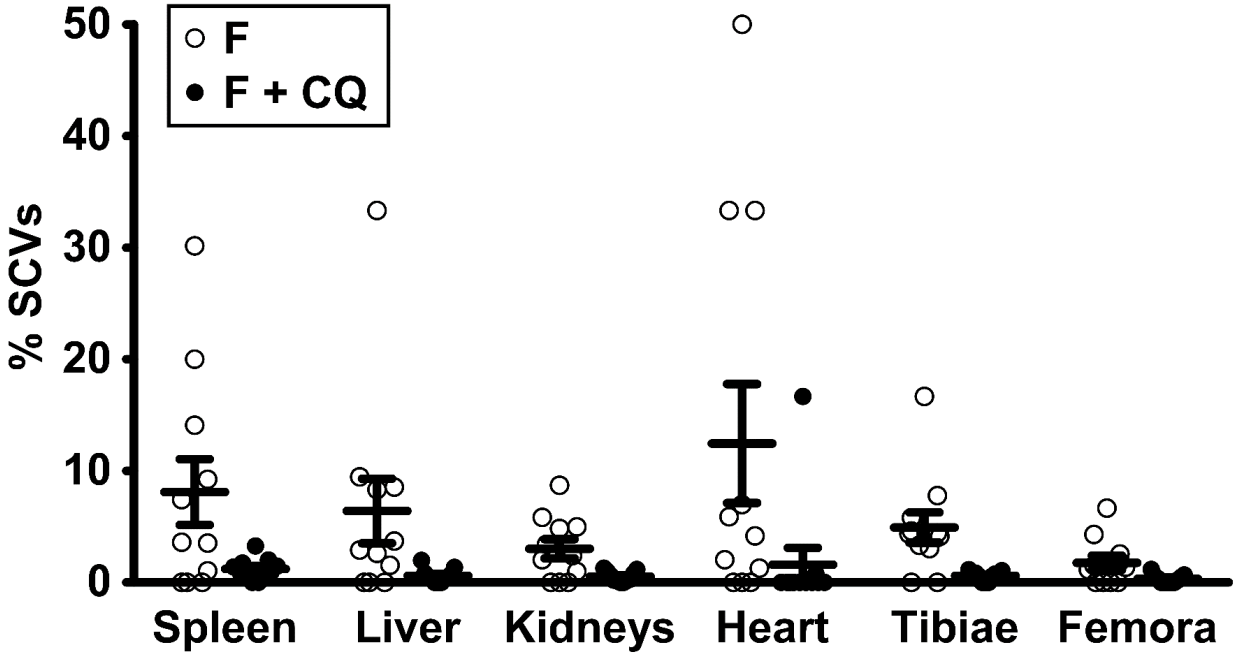


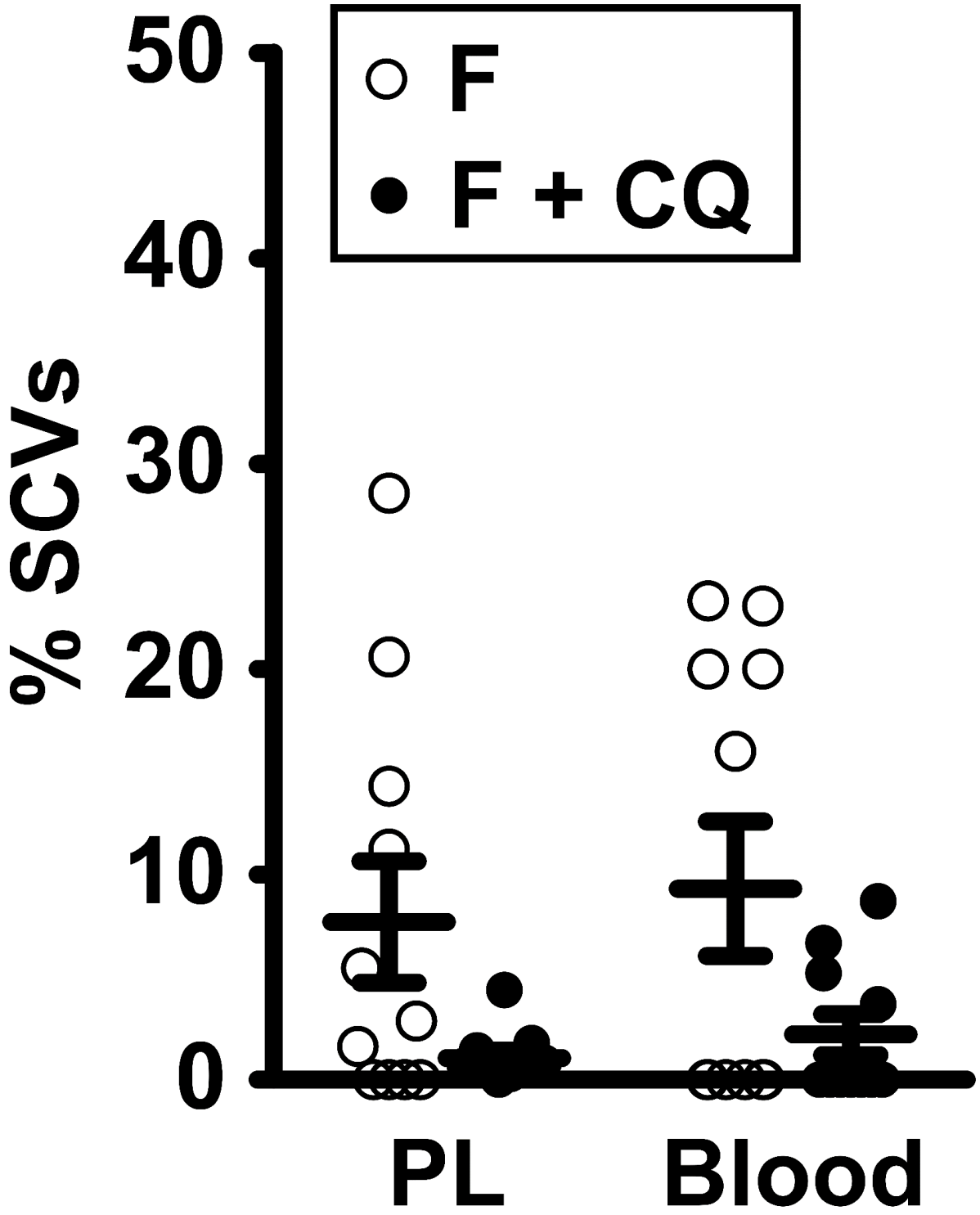












Tables

Table 1. *S. aureus* strains used in this study

Strain	Properties	Reference or source
6850	MSSA, WT isolate from osteomyelitis, strongly invasive, highly cytotoxic	ATCC 53657 [49]
JE2	CA-MRSA USA300, WT	NARSA[1]
Cowan I	MSSA, WT isolate from septic arthritis, strongly invasive, reduced cytotoxicity	ATCC 12598 [50]

[1]NARSA, Network of Antimicrobial Resistance in *Staphylococcus aureus*.